



II Simposio Radicales Libres y Antioxidantes

Facultad de Ciencias Químicas y Farmacéuticas
Universidad de Chile
Viernes 5 de diciembre de 2011

LIBRO DE RESUMEN

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FRAG-Chile, Free Radicals and Antioxidants Group, Chile

Temas y Expositores

- **ROS Y APOPTOSIS**

Dr. Sergio Lavanderos (U. Chile)

Dr. Andrew Quest (U. Chile)

- **METALES Y ESTRÉS OXIDATIVO**

Dr. Tulio Nuñez (U. Chile)

Dr. Pablo Muñoz Carvajal (U. Valparaíso)

- **CONFERENCIA PLENARIA**

DR. HOMERO RUBBO (URUGUAY)

Formación y Efectos Biológicos de Nitrolípidos.

ESTRÉS OXIDATIVO EN VEGETALES

Dra. Loreto Holuige (PUC)

Dra. Loretto Contreras (UNAB)

PRESENTACIONES ORALES

ORGANIZADORES

Alexis Aspée, *Universidad de Santiago de Chile*

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Claudio Olea, *Universidad de Chile*

Victoria Velarde, *P. Universidad Católica de Chile*



I Simposio “Radicales Libres y Antioxidantes”

Viernes 3 de diciembre de 2010
Empresa Portuaria Valparaíso

PROGRAMA

09:00 – 09:30 Inscripción

09:30 – 10:30 ROS y Apoptosis

Expositores Dr. Sergio Lavanderos (U. Chile)
Dr. Andrew Quest (U. Chile)

10:30 – 11:30 Metales y estrés oxidativo

Expositores Dr. Tulio Nuñez (U. Chile)
Dr. Pablo Muñoz Carvajal (U. Valparaíso)

11:30 – 12:00 Café

12:00 – 13:00 Conferencia

Dr. Homero Rubbo (Uruguay)
Formación y Efectos Biológicos de Nitrolípidos.

13:00 – 15:00 Almuerzo

15:00 – 16:00 Estrés oxidativo en vegetales

Expositores Dra. Loreto Holuige (PUC)
Dra. Loretto Contreras (UNAB)

16:00 – 17:00 Presentaciones Orales

Coordinador : Dr. Claudio Olea

17:00 – 19:00 Presentación de pósters

19:00 – 19:30 Premiación y Cierre



RESUMENES

PRESENTACION DE POSTERS

1. LACTATE EFFECT ON MITOCHONDRIA AND GLUCOSE UPTAKE IN HUMAN CANCER CELLS

Ahumada V1,2, Acuña-Castillo C1, Miranda D2, Montoya M1

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Cancer cells show an increase in glycolytic rate and in the production of lactate which correlate with poor prognosis. Recently, it was shown that lactate can be used as an energy molecule by aerobic cancer cells. Previously, we showed that mitochondria of cancer cell express proteins necessary for lactate oxidation, suggesting that lactate can be metabolized in this organelle. We postulate that lactate can modulate glucose metabolism of tumor cells, decreasing glucose uptake and increasing mitochondrial mass and/or membrane potential and ROS generation.

To analyze this hypothesis we incubated MDA-MB-231 and Caco-2 cells with different lactate concentration during 96 hours and then analyzed for mitochondrial mass (MitoTracker® Green FM), mitochondrial membrane potential (JC1) and ROS (CM-H2DCFDA) by flow cytometry. Glucose concentration was measured spectrophotometrically. Mitochondrial morphology was observed by transmission electron microscopy.

In MDA-MB-231 and Caco-2 cells, high concentration of lactate decreased glucose uptake and induced an increase in mitochondrial mass and ROS generation. Moreover, 20 mM of lactate also induced change in mitochondrial morphology. However, mitochondrial membrane potential was not altered in our experimental conditions.

These results suggest that lactate induce change in cancer cells metabolism, increasing mitochondrial activity. We can speculate that changes in mitochondrial activity might be related to poor prognosis in patients with increased lactate production.

Financial support: Proyecto Bicentenario PDA-20 (Conicyt) and DICYT-USACH.

2. GENERATION A MODEL OF OXIDATIVE STRESS INDUCTION USING ZEBRAFISH AND EVALUATION OF ANTI-OXIDANT, PROTECTIVE AND REGENERATIVE ACTIVITY OF RESVERATROL.

Alvarez M1, Egaña T2, Allende ML1

1. FONDAPE Center for Genome Regulation, Facultad de Ciencias, Universidad de Chile. 2. Department of Plastic Surgery and Hand Surgery, Faculty of Medicine, Technical University of Munich. Munich, Germany.

Introduction: Oxidation is biochemical process of electron loss always associated with another of uptake called reduction. Oxidation is essential for life, but an excess of oxidation in cells leads to oxidative stress, with toxic effects on different biological systems. We seek to generate an animal model of oxidative stress induction and to link oxidative status with regenerative capacity.

Material and Methods: We use larval and adult zebrafish of diverse transgenic strains to monitor cell survival and behavior. Oxidative stress is induced by exposure to cigarette smoke derivatives or by exposure to copper dissolved in water. As an antioxidant, we use resveratrol treatments in larval fish.

Results: Using different transgenic strains, we have evaluated the effects of cigarette smoke on cell death, angiogenesis and regeneration, we have shown cigarette smoke induces oxidative stress. Using a cell death and regeneration protocol by CuSO₄, we have used another transgenic line to evaluate the protective effect of the polyphenolic molecule resveratrol (3,5,4'-trihydroxy-trans-stilbene), known to promote cell survival and regeneration. Finally, we have measured the effect of cigarette smoke exposure on regeneration speed in adult fish.

Discussion: Our strategy will improve our ability to evaluate the effects of oxidative stress as well as to measure the antioxidant activity of different molecules in vivo using zebrafish as a bioassay.

Grant sponsors: FONDECYT 1110275; FONDAPE 15090007; CORFO-Innova (09MCCS-6705).

3. OXIDATIVE DAMAGE INDUCED BY HYDROPHILIC PEROXYL RADICAL ON PROTEINS PRESENT IN OCULAR GLOBE.

Arenas A.1, López C., Lissi E.3 and Silva E.1

1Departamento de Química Física, Facultad de Química, PUC, Chile; 2Departamento de Química y Farmacia, Facultad de Química, PUC, Chile; 3Departamento de Ciencias del Ambiente, Facultad de Química y Biología, USACH, Chile.

In this study we determine the damage caused by the interaction between free radicals and proteins present in the ocular globe. The studied proteins are lysozyme (LYSO), glucose 6 phosphate dehydrogenase (G6PD) and bovine lens protein (BLP). These proteins were exposed to peroxy radical obtained by the thermolysis of the azo compound 2,2'-azobis-(2-amidinopropane) (AAPH). The oxidative damage is classified in three categories: (i) changes in the SDS-PAGEs pattern. We found the formation of protein crosslinking, together with protein fragmentation in a lesser extent. (ii) Oxidative damage, evaluated through the measurement of carbonyl and peroxides groups. The AAPH treated proteins did not possess a large amount of carbonyl groups, which means that in these systems there are no long intercatenary reaction chains. The determination of total peroxides by Fox's assay, indicated a predominance of organic peroxides and a low concentration of hydrogen peroxide. (iii) Amino acids composition determination of the AAPH treated proteins shows only the modification of those residues, which are more sensitive to oxidation processes mediated by peroxy radicals. Tryptophan residues were the most damaged and the extension of this modification is large enough to justify its participation in the observed protein crosslinking. The presence of bityrosine in all AAPH incubated proteins was also studied, but the rather small amount of this compound found in all the cases do not allow to explain the observed crosslinking for each one of the studied proteins.

Acknowledgments: FONDECYT (Grant 1070285) and CONICYT (AT 24100085)

4. EFFECT OF HYDROPHILIC GRADE ON THE ANTIOXIDANT ACTIVITY OF PLANT RESINOUS EXUDATE, USING ASSAY ORAC-PGR

Chávez M, Modak B, Campos A.

Universidad de Santiago de Chile

We compared the antioxidant activity of resinous exudate of *Heliotropium sinuatum* and *Heliotropium stenophyllum* and its major flavonoids, pinocembrin and 3-O-methylgalangine, using the ORAC method in absence (ORAC-PGR) and presence of micelles of Triton X-100 (ORAC-PGRMIC). This method allows analysis of resinous exudate and flavonoids, through the protective effect of pyrogallol red (PGR) consumption induced by peroxy radicals derived from thermal decomposition of 2,2'-azobis-2-amidine propane (AAPH) in the aqueous phase. The presence of micelles decreased PGR protection elicited by both plants resinous exudates, as well as isolated flavonoids, in absence of micelles. This result has been interpreted in terms of PGR oxidation reaction taking place in the aqueous phase and partition of the active components in the resinous exudates and flavonoids in the Triton micelles. This is in agreement with experiments evaluating the protection of antioxidants remaining in the aqueous phase after extraction with octanol.

The results obtained in this work show that the antioxidant activity relates with the hydrophobicity and depends on the medium in which the reaction takes place, as well as of the location of antioxidants.

5. RESPONSES OF COLOBANTHUS QUITENSIS (KUNTH) BARTL. TO COPPER AND ALUMINUM STRESS IN VITRO.

Contreras, R.A.; Pizarro, M.; Köhler, H. & Zúñiga, G.E.

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Colobanthus quitensis (Kunth) Bartl., is the unique dicot plant that growth in the Maritime Antarctic territory. Our group has developed an in vitro culture system of plants, which allows us to study responses to different environmental conditions. In vitro plants were subjected to excess of copper (150 and 300 μ M) and aluminum (50 and 100 μ M), to evaluate membrane damage and antioxidant activity. Both metals increase the level of reactive oxygen species (ROS) and lipoperoxides. In addition, the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidases (POD) enzymes increased significantly in response to both elicitors. Furthermore, increased the content of phytochelatin and glutathione, metabolites that act as chelating agents to detoxifying the high concentrations of metals in cytosol. Finally, the presence of copper and aluminum inhibits the Asc-GSH cycle. Then, it is concluded that the mechanisms mediated by the enzymes SOD, CAT and POD, contribute to maintaining the redox status in a reducing condition.

Acknowledgements: To CONICYT and its program of doctoral scholarship.

6. EFFECT OF THYROID HORMONE (T3) ON BIOTRANSFORMATION PHASE 2 AND 3 PROTEIN EXPRESSION IN RAT LIVER.

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L-3,3',5-triiodothyronine (T3) induced liver preconditioning is associated with enhanced oxidative stress status. Nrf2, cytoprotective transcription factor controlling the expression of antioxidant cellular components, detoxification enzymes and membrane transporters. The aim of this study was to investigate whether T3 activates redox sensitive transcription factor Nrf2 and effect over biotransformation xenobiotic enzymes phase 2 and 3. Male Sprague-Dawley rats received a single ip dose of 0.1 mg T3/kg or T3 vehicle (controls) and nuclear and cytosolic protein levels of Nrf2 (Western blot) were determined at different times after treatment, in addition to those of Glutathione-S-transferases Ya and Yp (GST-Ya, GST-Yp), NADPH: quinone oxidoreductase-1 (NQO-1), epoxide hidrolase (EH), MRP2, MRP3 and MRP4 controlled by Nrf2. T3 administration produced decreases in hepatic cytosolic Nrf2 protein levels and increases in those of nuclear Nrf2 at 1 to 2 h compared to controls ($p < 0.05$). Liver levels of GST-Ya, GST-Yp, NQO-1, EH, MRP2, MRP3 and MRP4 exhibited a similar kinetic pattern with peak values at 1- 4 h after T3 compared to controls ($p < 0.05$). We conclude that administration of T3 activated Nrf2 at early times, consistent with increased biotransformation phase-2 and 3 protein expressions, thereby giving greater potential detoxifying basic mechanisms associated with T3-induced liver preconditioning. (FONDECYT 1090020)

7. ELECTROCHEMICAL, SPECTROSCOPY AND BIOLOGICAL STUDIES OF OXISOAPORPHINES FAMILY

C.Farias1,2, V.Castro1, C.Olea-Azar2, J.Maya3

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Some oxisoaporphines have been isolated from the rhizome of *Menispermum dauricum* DC, which occurs widely in the People's Republic Of China. The rhizomes of the plant are used in traditional Chinese medicine as an analgesic and Antipyretic. Preliminary studies of some analogs Oxisoaporphine, showed cytotoxic activity in cancer cells.

A new Family of Oxisoaporphine have been synthesized and studied in chemical and biological aspects. Given the structural similarity to oxidative stress-generating compounds, these have been proposed for the treatment of diseases such as Chagas disease, Cancer, etc.

In this work we studied the family of oxisoaporphine oxidized by electrochemical techniques. The cyclic voltammetry was carried out in aprotic medium. The voltammograms showed two quasireversible reduction couples. The reduction potentials varied depending on the structure of the oxisoaporphine. The Reduction mechanism proposed for such molecules involves the formation of radical species, which was detected by electron spin resonance (ESR). ESR spectra were well resolved and hyperfine pattern indicates that the unpaired electron is fully delocalized heterocycle, the results corroborate the mechanism proposed by cyclic voltammetry.

The cytotoxicity of this family found to be higher than Nifurtimox (a drug used clinically in Chagas diases) studies conducted in RAW 264.7 cell line. On the other hand are evaluating toxicity in tumor cells and *Trypanosoma cruzi*

PROYECTO PIA-ANILLOS ACT-112, FONDECYT 1090078.

8. ROLE OF COBALAMIN IN OXIDATIVE STRESS RESPONSE OF THE ACIDOPHILIC BIOLEACHING BACTERIUM *Leptospirillum ferriphilum*

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Leptospirillum ferriphilum is a chemolithoautotrophic bacterium that obtains its energy through oxidation of ferrous iron. This bacterium is an important bioleaching microorganism that participates in the recovery of metals from sulfide ores. Acid bioleaching environments are characterized by high levels of iron and heavy metals, which can increase the generation of reactive oxygen species (ROS) and induce oxidative stress to the microorganisms. Metaproteomic studies have shown that *Leptospirillum* spp. has high levels of antioxidant proteins and proteins related with cobalamin biosynthesis. Cobalamin is a cobalt-coordinated tetrapyrrole, which participates as a cofactor of many enzymes. Also has been described that cobalamin exerts a protective effect on oxidative stress in eukaryotic cells. This facts, leads us to propose that cobalamin plays a key role in the response to oxidative stress in *L. ferriphilum*.

In this study we evaluated the effect of cobalamin on the growth and intracellular ROS levels in *L. ferriphilum* cultured under oxidative stress conditions with Fe³⁺ and H₂O₂. Because cobalamin biosynthesis is limited by the presence of cobalt, we evaluated the effect of cobalt on bacterial growth. Our results shown that the cobalamin (5-10 nM) increased bacterial survival and reduced intracellular ROS levels of both culture subjected to oxidative stress and control. Similarly we determined that the cobalt (5-15 nM) increased the survival of bacterial cultures subjected to oxidative stress and control.

Altogether these results suggest that cobalamin may be involved in the antioxidant response of *L. ferriphilum*.

Acknowledgments. FONDECYT 11085045; 1110203; CONICYT doctoral fellowship

9. ANTIOXIDANT CAPACITY STUDIES AND BIOLOGICAL ACTIVITIES OF NEW COUMARIN-CHALCONES HYBRIDS

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Chalcones and coumarins are a family of natural and synthetic compounds that have been a great interest in because they possess varied pharmacological properties like antiparasitic and antioxidant properties. In this work we studied the antiparasitic activities of coumarin-chalcones hybrids, in *Trypanosoma cruzi*. Also, their mechanisms of oxidation were studied using cyclic voltammetry, since they might possess capacity scavenging free radical across processes of electronic transfer. In addition, we study their antioxidant capacity across the EPR spin-trapping studies of the hydroxyl radical scavenging (OH•). This study is based on the competence between a catcher agent (spin-trap) and the compounds in study for the hydroxyl radical. A screening was realized by a series of six hybrids chalcona-cumarina, in epimastigotes of *Trypanosoma cruzi* Dm28c which presented pronounced trypanocidal activities. On the other hand, the study of the mechanisms of oxidation of the compounds was realized in 75mM phosphate buffer (pH 7.4), and the results showed that these compounds present irreversible oxidation processes, with low potentials of oxidation, corroborating their potential use as antioxidant, and in the hydroxyl radical scavenging assay was observed an important decrease of the intensity of the sign of the spin-ducto after added the compounds in study.

Roberto Figuroa agradece Beca Doctoral Conicyt-Chile y proyecto Anillos ACT112

10. FIRST COMPREHENSIVE WEB-BASED DATABASE ON ANTIOXIDANTS IN FRUITS PRODUCED AND/OR CONSUMED WITHIN THE SOUTH ANDES REGION OF AMERICA

Fuentes J, Sandoval-Acuña C, Gómez M, Speisky H, Laboratory of Antioxidants, Nutrition and Food Technology Institute (INTA), University of Chile, Santiago, Chile.

A high consumption of fruits, particularly those rich in antioxidants, has been strongly associated with a lower risk of developing cardiovascular diseases and some forms of cancer. The latter has led to the construction of important databases on antioxidants in foods within Europe and the USA. Here, we report the first comprehensive web-based database on antioxidants contained in fruits produced and/or consumed in Chile and within the south Andes region of America. The database (available in www.portalantioxidantes.com) contains over 500 total polyphenol (TP) and ORAC values, for more than 140 species and/or varieties of fruits. All data presented in this database were generated from analysis conducted by a single ISO/IEC 17025-certified laboratory, using standardized TP and ORAC methodologies, and following a two-year systematic fruit-sampling program. Likewise observed in other databases, berries in general, walnuts, some prunes, cherries and apples, ranked among the richest antioxidant-containing fruits. The characterization also comprised native berries such as maqui (*Aristotelia chilensis*), murtila (*Ugni molinae*) and calafate (*Barberis microphylla*), which outscored most other fruits. Major differences in TP and ORAC values were observed as a function of the fruit variety in berries, avocado, cherries and apples. Our study also reveals that, in some fruits, a significant part of the TP and ORAC is accounted for by the antioxidants present in their peel. These data will be useful to estimate the fruit-based intake of total polyphenol (and through the ORAC, the antioxidant-related contribution) in the Chilean and other vicinal populations for its associations with health and diseases.

Funded by Corfo-Innova, 08CT11IUM-12.

11. GENERATION OF SUPEROXIDE RADICALS BY THE INTERACTION BETWEEN MERCURY IONS AND ENDOGENOUS THIOLS.

González C, a Speisky H, b, c Berrios T, a Vásquez-Arce A, a López-Alarcón C, a Olea-Azar C, c Barriga G, c and Aliaga ME. a Facultad de Química, Pontificia Universidad Católica de Chile, a Food Technology Institute and Faculty of Chemical and Pharmaceutical Sciences, University of Chile. c

The interaction between Hg²⁺ ions and endogenous thiols (RSH), such as: glutathione (GSH), cysteine (Cys), homocysteine (Hcy), cysteinylglycine (CysGly) and γ -glutamyl-cysteine (γ -GluCys), leads to the swift formation of the Hg(II)-thiol complexes. We investigated the potential capacity of Hg(II)-[RSH] and Hg(II)-[RSH]₂ complexes to reduce oxygen into superoxide radicals.

Among these complexes, only Hg(II)-[GSH]₂, Hg(II)-[Cys]₂ and Hg(II)-[Hcy] were able to reduce oxygen in a concentration-dependent manner. The generation of the superoxide radicals induced by these complexes was confirmed by EPR spin-trapping. Further evidence on the ability of the complex to generate superoxide radicals was attained through the demonstration of its capacity to reduce cytochrome c and/or to oxidize dihydroethidium (two superoxide-susceptible probes); being both effects SOD-inhibitable.

In the case of the solutions containing either Hg(II)-[CysGly]_n or Hg(II)-[γ -GluCys]_n (n = 1 or 2) no redox activity toward molecular oxygen was observed.

The incubation of the complexes Hg(II)-[GSH]₂, Hg(II)-[Cys]₂ and Hg(II)-[Hcy] with oxygen during 60 min led to the hydrogen peroxide-dependent oxidation of acetaminophen (e.g., CAT-inhibitable) and an increment in the formation of their oxidized thiols (oxidized glutathione, cystine and homocystine, respectively).

Thus the ability of the complexes to generate superoxide anions and, thereby, to deleteriously affect superoxide-susceptible biological targets warrants further studies.

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12. CHANGES IN LEVELS OF HEPATICS 8-ISOPROSTANES, OXIDIZED PROTEIN AND GLUTATHIONE IN OBESE MICE DIETARY SUPPLEMENTED WITH N-3 LONG CHAIN POLYUNSATURATED FATTY ACIDS

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Introduction: A high fat saturated diet produces a nutritional imbalance that could lead to obesity and subsequent triggering of metabolic syndrome, characterized for insulin resistance, oxidative stress and inflammation. N-3 long-chain polyunsaturated fatty acid such as both eicosapentaenoic (EPA) and docosahexaenoic (DHA) have anti-inflammatory and insulin-sensitizers properties, stimulate beta oxidation and inhibit lipogenesis, which transforms them (EPA and DHA) into protective agents against damage induced by chronic intake of high fat diet. Objective: To evaluate changes in hepatic oxidative stress parameters induced by a high fat diet along with a dietary supplementation with EPA+DHA. Materials and Methods: C57BL/6J male mice (n=9-13 per group) were fed for 12 weeks with (i) control diet (20% protein, 70% carbohydrate, 10% lipids), (ii) control plus EPA+DHA diet (200mg/Kg/day), (iii) high fat diet (20% protein, 20% carbohydrate, 60% lipids) and (iv) high fat plus EPA+DHA. We assessed insulin resistance (HOMA-IR), steatosis and oxidative stress (total glutathione, oxidized proteins and 8-isoprostane) hepatics. Results: Group (iii) showed significant increases (one-way ANOVA and the Newman Keuls test, p<0.05) in insulin resistance and oxidative stress parameters; those effects were reversed respect to control values in the (iv) group. Conclusion: Dietary supplementation with EPA+DHA in a high-fat diet prevents the induction of oxidative stress in experimental animals.

FONDECYT 1110043

13. ANTIOXIDANT CAPACITY STUDIES OF CHALCONES-COUMARIN DERIVATES.

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2Facultad de Farmacia, Universidad de Santiago de Compostela, España.

The human body has developed defense systems that maintain a balance between the amount of reactive species and antioxidants. However, when we break this balance against oxidative stress, which is why the presence of endogenously and / or exogenous antioxidants becomes essential in maintaining this balance. Among the coumarins are exogenous antioxidants which are a group of active principles derived from cinnamic acid with a skeleton benzo- α -pyrone, and chalcones a family of molecules derived from coumarin, its structure consists of two aromatic rings of which are linked by a carbonyl α , β -unsaturated three-carbon. To determine the antioxidant capacity assays were performed fenton competition in non-catalytic system, ORAC-FL and inhibition of ROS in a biological environment. Concluding that the derivatives have a high reactivity, as well as a good antioxidant capacity compared with trolox. Moreover, a study for electrochemical oxidation potentials which showed a similar behavior, where its potential is attributed to oxidation of the hydroxyl groups to the corresponding ketone derivatives. By studying ROS scavenging capacity in a biological system, correlation was found between the ORAC index and the percentage of inhibition, indicating that the main antioxidant mechanism of action is through the donation of a hydrogen atom (HAT).

Agradecimientos: Proyecto Anillos ACT-112

14. EVALUATION OF THE ANTIOXIDANT CAPACITY OF THREE PRODUCTS BASED ON THE PEDIAL SECRETION OF HELIX ASPERSA MÜLLER.

Herrera B, A C. Laboratorio Radicales Libres, Universidad de Valparaíso. The common snail is a gastropod with calcium shell in a spiral, extended body and cephalic tentacles. To move itself requires the secretion of mucus or slime, which to solidify serves as a support which isolates the unfavorable environment (capping). The snail and its mucus secretion are applicable from immemorial time in folk medicine. The use of snail secretion pedial in cosmetics, dermatology and medicine has been, since then, constantly increasing. Supposedly, without previous studies published, three products made with pedial secretion may possess antioxidant properties, therefore, are measured by ORAC-PGR and DPPH techniques.

Keywords: land snail, pedial secretion, antioxidant capacity.

(Thesis of Master of Biological Sciences, Free Radicals in Biomedicine).

15. TIME OF COLD STORAGE AND USE OF ANTIOXIDANTS ON RAINBOW TROUT (*Oncorhynchus mykiss*, Walbaum, 1792) SEMEN: EFFECTS ON VIABILITY PARAMETERS.

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The milt cold storage implies potential alterations in its quality, because the main storage process generates free radicals that produce spermatozoa membrane lipids damage with the consequent motility and fertilizing capacity alterations. For decrease the free radicals damage the cells have antioxidant defenses (proteins, enzymes, and low molecular weight substances). The objective of the present study was to evaluate the time effect and different antioxidants prepared in spermatic diluents on viability of *O. mykiss* milt storage at 4° C.

The two-way ANOVA denoted that the time storage and antioxidant influence have significant effects separated or combined on viability parameters (spermatic motility, viability spermatic, proteins concentrations and superoxide dismutase enzymatic activity in seminal plasma), whereas only the storage time affected the fertilizing capacity and catalase enzymatic activity in seminal plasma. The result analysis can conclude that the antioxidant presence improves the viability of cool storage semen specially the transport conditions and the antioxidants allow the fecundity in spite of motility decrease.

Acknowledgements: FONDEF D06I1020 Project, Quetro S.A and Dropco S.A.

16. REACTIVE OXYGEN SPECIES AND NITRIC OXIDE PRODUCTION IN ECV-304 CELLS CULTURED WITH PLASMA OF PATIENTS WITH IMPAIRED GLUCOSE METABOLISM

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Glucose fluctuations lead to endothelial dysfunction by a reduced bioavailability of nitric oxide (NO) and increased reactive oxygen species (ROS) production. Currently there are no tests to detect early endothelial dysfunction in subjects with early alterations in glucose metabolism. The aim of this work was to determine the ROS and NO production in ECV-304 endothelial cells cultured with plasma of patients with oral glucose tolerance test (OGTT).

From 40 patients with OGTT, 28 patients no presented alteration of glucose metabolism (N) and 12 were classified as prediabetes (PD) with impaired fasting glucose or impaired glucose tolerance. ECV-304 cells were incubated with basal and postprandial plasma. ROS and NO production were measured with fluorescent probe and kinetic parameters were calculated: production maximum (IF/ug protein) and production rate (IF/ug protein*min).

Cells incubated with basal and postprandial plasma of N showed an increase in maximum ROS production compared to PD ($p < 0.05$). ROS production rate was higher for postprandial plasma in N compared with its basal plasma or PD plasmas ($p < 0.05$). However, ROS production rate according to HOMA-IR value (≥ 2.5 insulin resistance) was higher in subjects with insulin resistance in postprandial condition. PD showed increased NO synthesis in postprandial plasma compared with N plasmas ($p < 0.05$). In subjects N and PD or grouped by HOMA-IR value, the NO synthesis rates were lower for postprandial plasma compared to basal plasma in both cases.

The ECV-304 endothelial cells respond to glucose fluctuations in plasma of patients with OGTT through changes in ROS and NO production.

Acknowledgements: DIUC211.072.035-1

17. STUDIES OF DINITROINDAZOLES PYRIDINE AND TRIAZOLE AS POTENTIAL ANTICHAGASIC FAMILY.

Lapier M, Lopez-Munoz R, Aguilera B, Maya JD, Belen, Aran V, Olea-Azar C.

Program and Clinical Molecular Pharmacology, Institute of Biomedical Sciences (ICBM), Faculty of Medicine University of Chile. Santiago Chile.

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The American Trypanosomiasis is caused by the protozoan *Trypanosoma cruzi*. The use of drugs such as nifurtimox and benznidazole in the treatment of the Chagas disease has been limited by the frequent occurrence of adverse effects, which has led to research new pharmacological proposals. We propose that the new families and triazolo pyridines dinitroindazoles follow different mechanisms, but effective against *T. cruzi*.

Family was assessed by Cyclic Voltammetry and Electron Spin Resonance. Dinitroindazoles, showing one-electron reduction of the nitro group and a mechanism for autoprotonation. The nitro-radical was characterized by REE, showing the characteristic hyperfine pattern nitroindazoles. To relate the reduction mechanisms observed in biological systems the generation of ROS in parasites, which would possibly via generation of oxidative stress. On the other hand trypanocidal activity was quantified in both families, through studies of cell viability.

The results suggest that the family of dinitroindazoles follow a mechanism similar to nifurtimox and / or benznidazole via free radicals, because during the reduction of the nitro group nitro anion radical species generated. The triazolo pyridines family follows an alternative mechanism to the free radicals, possibly via enzyme inhibition. Both families were shown to be effective against *T. cruzi*, but to a lesser degree than nifurtimox.

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18. PHENOLIC COMPOUNDS IN PLASMA AFTER RED GRAPE JUICE INTAKE.

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The aim of this study was to describe the bioavailability of major phenolics in Autumn Royal red grape juice: catechin, gallic acid, and malvidin. Sixteen healthy adult subjects were distributed in two groups: experimental (n=8) receiving 300 mL grape juice, and placebo (n=8) receiving 300 mL artificial beverage. Blood was obtained before (t=0) and after 30, 90, 180, and 300 min since juice or placebo intake. The analysis of phenolics in plasma was performed by HPLC using UV detection at 280 nm. At 30 min, plasma catechin and gallic acid levels were 0.92±0.02 and 0.06±0.01 µg/mL, respectively (p<0.01). No phenolics were detected in plasma of the subjects of the placebo group. At 180 min, catechin and gallic acid levels were 2.06±0.15 and 0.26±0.01 µg/mL (p<0.01). After 300 min, gallic acid was not detected in plasma and only two subjects exhibited measurable levels of catechin. Malvidin was not detected. After the intake of a regular serving of grape juice antioxidant phenolics are bioavailable and thus potentially capable of exerting their expected biological effects. In conclusion, our data indicate that plasma catechin and gallic acid levels, although low, are still able to contribute to the total antioxidant body status.

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19. REACTION OF GLUTATHIONE WITH GALVINOXYL AND DPPH FREE RADICALS IN MICELLES

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Reaction of alkanethiols with stable free radicals galvinoxyl and DPPH in homogeneous media showed little influence of the organic solvent employed. This result contrasts with the typical solvent effect described for phenols. In fact, the rate reactions for alkanethiols were faster in ethanol than in apolar organic solvents and they decreased in acid organic media. This behavior has been interpreted in terms of an electron transfer reaction from the ionized thiol moiety toward the free radicals.

In spite of the compartmentalization of DPPH and galvinoxyl in micelles, and the preferential location of the hydrophilic reduced glutathione in the aqueous phase, it was observed relatively fast reactions in micelles, increasing with the concentration of GSH. Evaluation of the consumption of the free radical by the presence of an excess of the glutathione relative to the free radical permitted to define an observed rate constant (kobs). These kobs constants increased with the concentration of GSH reaching a plateau that was dependent on the concentration of the surfactant employed. Moreover, data evaluate under similar experimental conditions allowed us to establish rate reactions following this trend for the micelles: CTAC > Triton X100 > SDS.

This complex kinetic observed in micelles has been explained in terms of changes on the media pH, partition of the GSH in neutral micelles and/or in charged micelles as a counter ion, as well as, influence of consumption GSH in chain reaction.

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20. METALS POLLUTION AND ITS EFFECT OVER ANTIOXIDANT ACTIVITY OF CHILEAN BEE POLLEN SAMPLES

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Chile produces a variety of bee products with different botanical origins and several biological characteristics inherited from specific floral sources. Phenolic compounds are related to these biological activities. Plants and beehives in areas polluted with metals may increase the levels of these elements in honey and bee pollen. Thus, it is expected that the honey and bee pollen that are produced in such places have higher metal content, which permits their use as geographic indicators of environmental pollution by metals. Furthermore, all of these honey and bee pollen samples have modified biological properties, but their antioxidant activities are determined by their botanical origin and metal content. The bee pollen samples produced near the zones with potential pollution by metals from Central and Southern Chile were studied to determine their metals content as well as their antioxidant activities. Depending of the geographical origin, samples from Central Chile showed increased levels for lead, cadmium and iron, whereas bee pollen originated in Southern Chile showed increased levels for copper and iron.

The selected bee products herein had higher metal content as well as lower phenolic content and antioxidant activity as determined by FRAP and DPPH assays compared with control samples.

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21. KINETIC CHANGES OF LIVER Nrf2 ACTIVITY INDUCED BY IRON (Fe) ADMINISTRATION.

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Fe catalyzes redox reactions and triggers cellular oxidative stress (OXE). Protection against ischemia-reperfusion injury in the rat is afforded by a subchronic Fe protocol (6 doses, 50mg/kg, i.p. on alternate days), in concomitance with the induction of transient/moderate OXE. In this respect, it is important to consider the participation of the redox-sensitive transcription factor Nrf2, which regulates the expression of anti-oxidant cytoprotective proteins. Under homeostatic conditions, Nrf2 is bound in the cytoplasm to its inhibitor Keap-1 (Nrf2/Keap-1 complex). Under transient/moderate OXE conditions, the Nrf2/Keap-1 complex is dissociated and Nrf2 is translocated to the nucleus and bound to DNA specific sequences (ARE).

In the present study, we assessed kinetic changes in liver OXE [total equivalents of reduced glutathione (GSH)] in relation to the nuclear translocation of Nrf2 (kinetic changes in nuclear Nrf2/Keap-1 complex), after a subchronic Fe protocol (6 doses of 50mg/kg ip on alternate days). Male Sprague-Dawley rats received Fe or saline (controls), and assessments were carried out 24 h after each Fe dose (1-6 Fe doses). Liver GSH was assessed spectrophotometrically and the nuclear contents of Nrf2 and Keap1 were evaluated by westernblot.

22. COMPARATIVE EVALUATION OF OXIDATIVE STRESS IN SCYTOSIPHON LOMENTARIA (PHAEOPHYTA), FROM MINING SECTORS.

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The increasing pollution of aquatic coastal ecosystems, by trace metals has led to increased interest in determining its effects over the organisms living in these environments. Seaweeds are known for their ability to bioremediate, for this reason it is possible that bioavailable trace metals in the environment, can be absorbed and therefore generate oxidative stress.

Samples were collected from Atacama region areas due to important mining activity in Caleta Palito, Chañaral and Huasco, in comparison with the sector Quintay (R. Valparaíso) with less antropogenic activity. Metal concentrations of copper and iron analyzed, indicated that Caleta Palito presents higher values than Huasco, Chañaral and Quintay.

Scytosiphon lomentaria's samples were collected for analysis of damage to lipids (TBARS), antioxidant enzymes's activity (catalase and superoxide dismutase) and total antioxidant capacity (TRAP). Samples from Caleta Palito and Chañaral have high rates of oxidative stress : increased enzymatic activity, increased oxidative damage and low values of antioxidant capacity. Moreover, Huasco, considered an area impacted by mining, the results show that samples have a comparable oxidative stress to Quintay.

The results demonstrated that the specie Scytosiphon lomentaria can be considered as a biomonitor of oxidative damage.

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23. ELECTROCHEMICAL, SPECTROSCOPIC AND BIOLOGICAL STUDY OF A NEW FAMILY OF 5-NITROINDAZOLES WITH POTENTIAL ANTICHAGASIC ACTIVITIES.

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Parasitic diseases are important in morbidity and generate great economic troubles in Third World countries and especially in very lower class of

the population. Nifurtimox, the drug used for the treatment of Chagas disease, is capable of producing free radicals, which in an aerobic environment yield, by recycling redox, reactive oxygen species(ROS) (which are highly reactive substances that can cause significant cellular damage). Recently electrochemical and spectroscopic studies have been performed in compounds with important biological activity. These compounds have been synthesized by structural modifications in its side chain to gain more potent activity and to improve their properties such as lipophilicity, reduction potentials, and so on. This work presents a study of a family of 5-nitroindazolones, in which their electrochemical behavior was characterized by cyclic voltammetry technique (where all the potentials of generation of the free radical were lower than nifurtimox, a drug used clinically). By electron spin resonance (ESR), free radical species, generated electrochemically and biologically, were studied. Biological measurements were performed in murine RAW 264.7 cells and in Dm28c strains of Trypanosoma cruzi in its epimastigote stage by means of cytotoxicity and parasitic inhibition techniques.

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24. FORMATION AND STABILITY OF CHLORAMINES

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In the last few years, the study of chloramines, formed by reaction of hypochlorous acid with primary amines, has been mainly focused on evaluating the oxidation of lipids, proteins, and even exogenous compounds including drugs and antioxidants. Chloramines once are formed they are relatively stable, and show longer lifetimes than other oxidizing species (such as oxygen or nitrogen free radical species) in biological systems. However, they are able oxidize reducing substrates such as phenols, and/or react by chlorination of aromatic compounds. In addition to that, chloramines can also extend the initial damage to biomolecules remote sites by radical reactions catalyzed by transition metals or by transchlorination. In particular, these processes depend on both the stability of chloramines and their microenvironment, and as well as to the presence of antioxidants able to interact with these species preventing the damage induce by chloramines. Recently, we have been studying the stability of various chloramines in order to desing models form evaluating kinetic reactivity of different antioxidants against these species and the damage to biomolecules such as proteins or lipidic membranes.

In this work, we study the formation of chloramines generated in situ by reaction of primary amines with hypochlorous acid and discuss their properties in comparison with model chloramines obtained by chemical synthetic methods. These studies involve the influence of the temperature and the presence of metals on the stability of different chloramines derived from free amino acids like lysine, alanine and a primary amine such as butylamine.

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25. REACTIVITY OF SOME SYNTHESIZED HYDROXYCOUMARINS WITH ELECTROGENERATED SUPEROXIDE ANION.

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Coumarins (2H-benzopyrone-2-ona) include a group of natural products of considerable importance, being widely distributed throughout the plant kingdom. Interest in the chemistry of this type of compounds arises from its diversity of pharmacological activities, which manifests particularly in the hydroxylated derivatives. Natural and synthetic coumarins possess anti-oxidant, anti-inflammatory, anticoagulation, and anticancer activities.

15 mL of an extra-dry DMSO solution at 20°C containing the supporting electrolyte 0.1 M TBAHFP was saturated by pure dry oxygen during 20 min. The cyclic voltammogram (CV) of the oxygen reduction on glassy carbon electrode was then recorded at a scan rate 0.1 V/s. The initial potential was fixed at -0.5 V vs. Ag/AgCl. The scanning potential was reversed at -1.0 V vs. Ag/AgCl. A stock solution of the coumarin or the standard antioxidant was prepared at about 0.1 M Aliquots of the stock solution were successively added to the 15 mL oxygen solution in order to get an antioxidant substrate concentration in the range (0.2-2.50 mM). After each aliquot addition, CV of the oxygen solution was recorded at a scan rate 0.1 V/s. From $I_{pa0} - I_{pas} / I_{pa0}$ vs coumarin concentration plots were calculated the AI50 Antioxidant Index.

The reactivity of mono- and dihydroxy-substituted coumarins towards superoxide anion ($O_2^{\cdot-}$) was assessed by a cyclic voltammetric methodology. From the results can be concluded that mono-hydroxy-substituted coumarins (4-methyl-7-hydroxycoumarin, 4-chloromethyl-7-hydroxycoumarin) were less reactive than that of dihydroxy substituted coumarins (4-methyl-7,8-dihydroxycoumarin, 4-chloro-7,8-dihydroxycoumarin). AI50 values for monohydroxy-substituted coumarins varied between 1.88 mM and 2.62 mM. However, dihydroxy-substituted coumarins were the most reactive compounds with AI50 varying between 0.99 mM and 1.65 mM. The standard antioxidant, Trolox, exhibited an AI50 of 1.91 mM. The presence in 4-position of chloromethyl moiety enhanced the reactivity towards the superoxide anion.

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26. ELECTROCHEMICAL STUDY AND ELECTRON SPIN RESONANCE (ESR) OF COUMARINS WITH BIOLOGICAL INTEREST

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Coumarins or 1-benzopin-2ona are present in nature and widely distributed in medicinal plants and some grains. These derivatives have attracted considerable interest because of its numerous biological activities, such as their anti-cancer, anticoagulants, antioxidants, among others.

This work will focus primarily on the study of a new family of coumarin derivatives through an electrochemical analysis and the characterization of the intermediate-radical oxidation mechanism of this new family, using Electron Spin Resonance spectroscopy.

In order to characterize the electrochemical behavior of the molecule SVR20, 21 and 41, cyclic voltammetry studies using a mercury drop were done. The results showed that the mechanism of reduction involves two quasi-reversible coupled throughout the speed range studied (100-2000mV/s). The first couple appears to approximate -0.6mV and the second at -0.9mV. These two couples were attributed to the formation of nitro radical anion derivative and the subsequent hydroxylamine derivative formation.

REE spectra confirmed the presence of radical species in the mechanism of reduction obtained well-resolved signals. The hyperfine coupling indicated that the deslocalization was in the coumarine ring. For molecules (d) and (e), we studied the oxidation mechanism using cyclic voltammetry using carbon vitreo electrode. We observed two of oxidation couples throughout the speed range used, which would be attributed to a semi-quinone species and the corresponding ortho-quinones. Intermediaries will be characterized by spectroscopy radicalarios REE.

27. SYNTHESIS, PARTITION AND REACTIVITY OF TEMPO DERIVATIVES OF DIFFERENT HYDROPHOBICITY WITH PYRANINE IN HOMOGENEOUS AND MICROHETEROGENEOUS MEDIA.

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The effect of free radicals in biological systems has been extensively studied, and how radicals can be distributed in two-phase systems (lipid /water). Experimentally this can be quantified by determining its partition constant which indicates the permeability and solubility in lipophilic systems.

In the present study we synthesized compounds based on the structure of radical TEMPO (1-oxy-2,2,6,6-tetramethylpiperidine) linked to alkyl chains of variable length: methyl, hexanoyl and dodecanoyl through an amide moiety in 4 position. Once obtained, the spin probes were evaluated in their capability to quench the fluorescence of a hydrophilic fluorophore such as pyranine. Stern-Volmer plots are obtained in homogeneous (acetonitrile) and microheterogeneous media, by employing a neutral surfactant (Triton X100 reduced) and above its cmc (0.2 mM).

We correlate the quenching experiments with results of molecular recognition or docking for these probes, giving us an idea of the insertion degree of these spin probes in micellar models. We calculated distribution constant for each compound by employing a methodology based on the evaluation of the g-factor EPR parameter at increased amount of surfactant concentration. We correlated the partition of the 3 spin probes with the quenching constant obtained for the reaction of these compounds with pyranine in microheterogeneous media.

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28. ROLE OF SUPEROXIDE ANIONS IN THE REDOX CHANGES THAT AFFECT THE PHYSIOLOGICALLY-OCCURRING CU(I)-GLUTATHIONE COMPLEX

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The physiologically occurring copper-glutathione complex, $[Cu(I)-[GSH]_2]$, has the ability to react continually with oxygen, generating superoxide anions ($O_2^{\cdot-}$).

$Cu(I)-[GSH]_2 + O_2 \rightarrow IOC + O_2^{\cdot-}$ (Rx. 1) (IOC= Intermediate Oxidized Complex)

Recent studies conducted by us suggest that removal of superoxide from Rx.1 may result in the formation of $Cu(II)-GSSG$.

$IOC + 2O_2^{\cdot-} \rightarrow Cu(II)-GSSG + H_2O_2 + O_2$ (Rx. 2)

In the present study we addressed the oxidizing effects that removal of superoxide from a solution containing the $Cu(I)-[GSH]_2$, induced by either temperature or addition of Tempol (a SOD-mimetic), would have on the redox state of the $Cu(I)$ ion and GSH molecules of such complex. Under such conditions, we also investigated whether $Cu(II)-GSSG$ is formed as a final oxidation product along time. In addition, we addressed the potential of a source of $O_2^{\cdot-}$ external to the $Cu(I)-[GSH]_2$ complex to prevent its oxidation. Removal of $O_2^{\cdot-}$ from a $Cu(I)-[GSH]_2$ -containing solution, whether spontaneous (T°) or Tempol-induced, led to time-dependent losses in GSH that were greater than those affecting the metal. The losses in GSH were not accompanied by increments in GSSG, but were largely accounted for by the cumulative formation of $Cu(II)-GSSG$ molecules. Notably, the redox changes in $Cu(I)$ and GSH were totally prevented when $Cu(I)-[GSH]_2$ was co-incubated with a mixture of hypoxanthine/xanthine oxidase. Data suggest that the generation of $O_2^{\cdot-}$ by $Cu(I)-[GSH]_2$ implies the obliged formation of an intermediate whose subsequent oxidation into $Cu(II)-GSSG$ or back-reduction into $Cu(I)-[GSH]_2$ is favored by either the removal or the addition of $O_2^{\cdot-}$, respectively. It could be speculated that due to the presence of various superoxide removing or reacting molecules, the $Cu(I)-[GSH]_2$ complex might occur within cells, not only as such but also as $Cu(II)-GSSG$, its end-oxidation product.

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29. USE OF ANTIOXIDANTS ON RAINBOW TROUT *Oncorhynchus mykiss* (Walbaum, 1792) SPERM DILUENT: EFFECTS ON MOTILITY AND FERTILIZING CAPABILITY.

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The present investigation determined how different antioxidants incorporated into the sperm diluent for cold storage of semen affected sperm motility and spermatozoan fertility capabilities of the rainbow trout. The diluents were prepared using a base of UCT diluents (T2), adding grape polyphenol (T3), trolox C (T4), polyphenol plus trolox (T5), and vitamin C (T6). The incorporation of antioxidants into sperm diluents prolongs motility and fertility of rainbow trout semen. The results show that by day two, all of the treatments showed level 5 sperm motility. After seven days of storage, only T3 and T6 dropped to level 4 sperm motility. The duration of flagellate activity on this day was maximal for T3 with 36.87 ± 0.51 s and minimal for T6 with 29.78 ± 0.52 s. On day seven, fertility was maintained with no statistically significant differences between the control and T2 ($92.80 \pm 0.62\%$), T3 ($83.66 \pm 2.52\%$), T4 ($90.46 \pm 1.60\%$), T5 ($83.57 \pm 2.75\%$), and T6 ($83.57 \pm 2.30\%$). By days 10 and 17 of storage, the fertility of T1 was zero and that of T2 was significantly lower than the control group. On day 17, the highest percentage of fertilization was $97.38 \pm 1.85\%$ for T5 and the lowest value was $64.69 \pm 3.76\%$ for T2. The results allow concluding that the sperm viability of semen stored with different antioxidants is significantly prolonged.

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30. DETERMINATION OF A NON-PRECONDITIONING PROTOCOL BY IRON AGAINST LIVER ISCHEMIA REPERFUSION INJURY

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Iron (Fe), an essential micronutrient catalyzes redox reactions thus inducing cellular oxidative stress (OXE). In this respect, subchronic treatment with Fe (6 doses, 50mg/kg, i.p. on alternate days) induces transient OXE in a time window of 72 h and preconditions the liver against ischemia-reperfusion (IR) injury in the rat. In order to generate preconditioning strategies induced by Fe administration plus other transient OXE inducing agents, we evaluated liver OXE (glutathione content) induced by 2 doses of Fe, in relation to hepatoprotection (serum transaminases GOT and GPT, and liver histologies), against IR injury (1 hour ischemia and 20 hours of reperfusion), in Sprague-Dawley rats, grouped as: (1) saline-sham laparotomy (controls); (2) saline-IR; (3) Fe-sham; (4) Fe-IR.

In relation to controls, significantly enhanced serum GOT [69.48%] and GPT [171.6%], with mild to moderate congestion, scattered cellular necrosis, and isolated neutrophil infiltration, were observed in the saline-IR group. Although the Fe protocol was able to induce reversible liver OXE, in a time window of 72 h, no liver protection was observed in the Fe-IR experimental group, as shown by enhancements of 55.11% (GOT) and 34.34% (GPT) and liver morphologies similar to those in the saline-IR group. Thus, we conclude that 2 doses of Fe constitute an adequate protocol to be used in conjunction with other liver preconditioning strategies triggering transient OXE, such as the administration of T3.

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31. VARIATION OF ANTIOXIDANT CAPACITY IN DIFFERENT STAGES PRODUCTION OF SAUVIGNON BLANC WINE

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Wine contains a great variety of known antioxidants such as polyphenols. These antioxidants, which are hydrogen donors, capture electron and free radicals and stop the oxidative process of wine.

These compounds are extracted in the maceration process. For white wines, this stage is about 6 hours, and the subsequent pressing of the grape also contributes with antioxidants in wine.

The white wines are characterized by low concentrations of polyphenols, due to the type of grape as well as the maceration time required. Therefore, its antioxidant capacity is lower than red wines. Because of this reason, the oxidation of white wines is highly controlled at different stages of production. Controlling white wine oxidation can be achieved using low temperatures throughout the winemaking process and / or applying inert gases, ascorbic acid, sulfuric acid, etc..

In this study, the variation in antioxidant activity of three Sauvignon Blanc wines from the Casablanca Valley were investigated using two different methods: the technique ORAC (Radical Absorbance Capacity Oxygen) with two different substrates: (i) fluorescein (ORAC-FL) and (ii) pyrogallol red (ORAC-PGR). The rate of reaction between the PGR and AAPH() is much faster than FL, so the second method gives information on the reactivity of antioxidants more than stoichiometric factors as fluorescein.

The results show no significant differences with the ORAC-FL method, unlike the antioxidant reactivity (ORAC-PGR) showing a significant and expected difference in the various production stages

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